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MicroReview

Lipid trafficking to the outer membrane of Gram-negative bacteria

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Summary

The envelope of Gram-negative bacteria is composed of two distinct lipid membranes: an inner membrane and outer membrane. The outer membrane is an asymmetric bilayer with an inner leaflet of phospholipids and an outer leaflet of lipopolysaccharide. Most of the steps of lipid synthesis occur within the cytoplasmic compartment of the cell. Lipids must then be transported across the inner membrane and delivered to the outer membrane. These topological features combined with the ability to apply the tools of biochemistry and genetics make the Gram-negative envelope a fascinating model for the study of lipid trafficking. In addition, as lipopolysaccharide is essential for growth of most strains and is a potent inducer of the mammalian innate immune response via activation of Toll-like receptors, Gram-negative lipid transport is also a promising target for the development of novel antibacterial and anti-inflammatory compounds. This review focuses on recent developments in our understanding of lipid transport across the inner membrane and to the outer membrane of Gram-negative bacteria.

Introduction

The envelope of *Escherichia coli* and other Gram-negative bacteria contains two distinct lipid bilayers that are separated by the peptidoglycan-containing periplasmic space. These are the inner membrane (IM) and the outer membrane (OM) (Duong *et al*., 1997; Raetz and Whitfield, 2002). This envelope is unique to Gram-negative bacteria and is the site of action of several classes of antibiotics. In addition, it provides a fascinating model of complex macromolecular assembly that has been explored using the tools of biochemistry, genetics and structural biology. In spite of this, many molecular details of the assembly of this structure remain obscure. This review will summarize recent developments in our understanding of the biogenesis of the Gram-negative cell envelope with special emphasis on mechanisms of lipid transport to the OM (Fig. 1).

The Gram-negative IM is composed of (glycerol-phosphate based) phospholipids in both its inner and outer leaflets as well as integral and peripheral membrane proteins. The IM carries out a variety of functions typically assigned to both the plasma membrane and specific organelles in higher organisms. These include protein export, solute import, cell signalling, biosynthesis, electron transport, and maintenance of a proton motive force and ATP synthesis.

The OM plays an important role in nutrient uptake but in addition provides the organism with a remarkable permeability barrier, conferring resistance to a variety of detergents and antibiotics. The OM is a unique asymmetric lipid bilayer consisting of an inner face of phospholipids and an outer face of lipopolysaccharide (LPS). LPS is a complex, glucosamine-based glycolipid unique to Gram-negative bacteria that is required for viability of most strains (Raetz and Whitfield, 2002) (Fig. 2). The hydrophobic anchor of LPS is termed lipid A, also known as endotoxin. Lipid A is a powerful activator of the mammalian innate immune response via its activation of Toll-like receptors (Poltorak *et al*., 1998; Beutler, 2004). Lipid A released by bacterial growth or death during infection can result in overactivation of these receptors causing monocytes and macrophages to release a number of proinflammatory cytokines leading, if untreated, to a condition termed Gram-negative septic shock (Riedemann *et al*., 2003). In addition to LPS, many enteric bacteria also have capsular polysaccharide, glycolipids containing phospholipid membrane anchors, present at the outer surface of the OM.

In addition to phospholipids and LPS, the Gram-negative OM also contains a unique set of transmembrane proteins that adopt a β-barrel architecture. This protein structure is not found in IM proteins but is found in the OM of the eukaryotic mitochondria and chloroplast (Wim-

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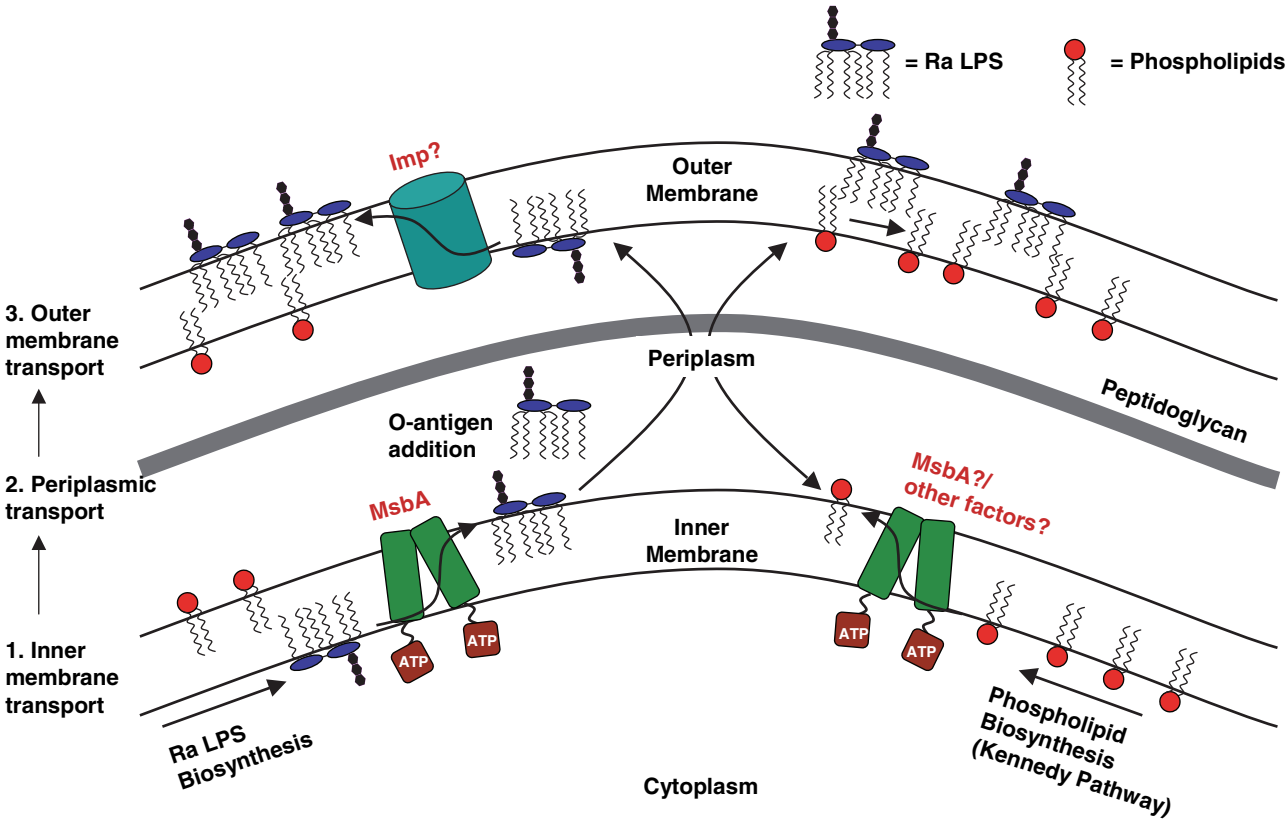

Fig. 1. Schematic showing steps of lipid movement through the envelope of *E. coli*. Phospholipids and Ra-lipid A are synthesized in the cytoplasm and at the inner leaflet of the IM using cytoplasmic enzymes and precursors. These molecules are then transported across the IM to achieve a periplasmic orientation. IM protein MsbA plays a major role in this process. MsbA is a homodimer of monomers containing a membrane domain (shown in green), a linker region, and an ATP binding domain (shown in brown). Some strains add O-antigen to Ra-LPS in the periplasm. LPS and a portion of the phospholipids must then be moved across the periplasm to reach the OM by yet unknown mechanisms. LPS is then oriented to the outer leaflet of the OM by a process that may involve the protein Imp/OstA (blue). Arrows refer to unidirectional or bidirectional transport of lipids.

ley, 2003). Many OM proteins carry out functions in nutrient transport into the cell; but some function in protein secretion, adhesion or catalysis. The only non-β-barrel proteins found in the *E. coli* OM are the lipoproteins that associate with the membrane via an N-terminal lipid modification (Tokuda and Matsuyama, 2004). OM proteins are synthesized at the cytoplasmic protein synthesis machinery, and then transported across the IM, before being incorporated into the OM. The export of OM proteins requires the IM Sec preprotein translocation apparatus and periplasmic folding factors such as SurA. Recent evidence has indicated that the OM protein Omp85/YaeT is required for proper export and oligomerization of OM proteins (Voulhoux *et al.*, 2003). Export and folding of OM proteins has been reviewed in marvellous detail elsewhere (Wickner *et al.*, 1991; Mori and Ito, 2001; Silhavy *et al.*, 2006) and is not considered extensively here except to point out that extracytoplasmic assembly of both proteins and lipids are required for proper function of the Gram-negative OM.

Membrane lipids and biosynthesis

The major membrane lipids of *E. coli* include LPS and the phospholipids phosphatidylethanolamine (PE) (Fig. 2), phosphatidylglycerol (PG) and cardiolipin. As mentioned above, phospholipids are found in both the IM and the OM. In contrast, essentially all of the LPS is found in the outer leaflet of the OM. The inner leaflet of the IM is the site of the late steps in the synthesis of phospholipids and LPS. Indeed, *E. coli* K-12, which produces an LPS without an attached O-antigen, synthesizes the entire LPS molecule in the interior of the cell (Fig. 1). This topology is inferred from both the predicted cytoplasmic location of enzyme active sites and of enzyme substrates (ATP, nucleotide sugars, acyl-ACPs, etc.).

The biosynthesis of phospholipids occurs via the Kennedy pathway (Cronan, 2003) and the biosynthesis of lipid A occurs via the Raetz pathway (Raetz and Whitfield, 2002). The structural genes for the enzymes involved in these pathways have been identified and characterized in

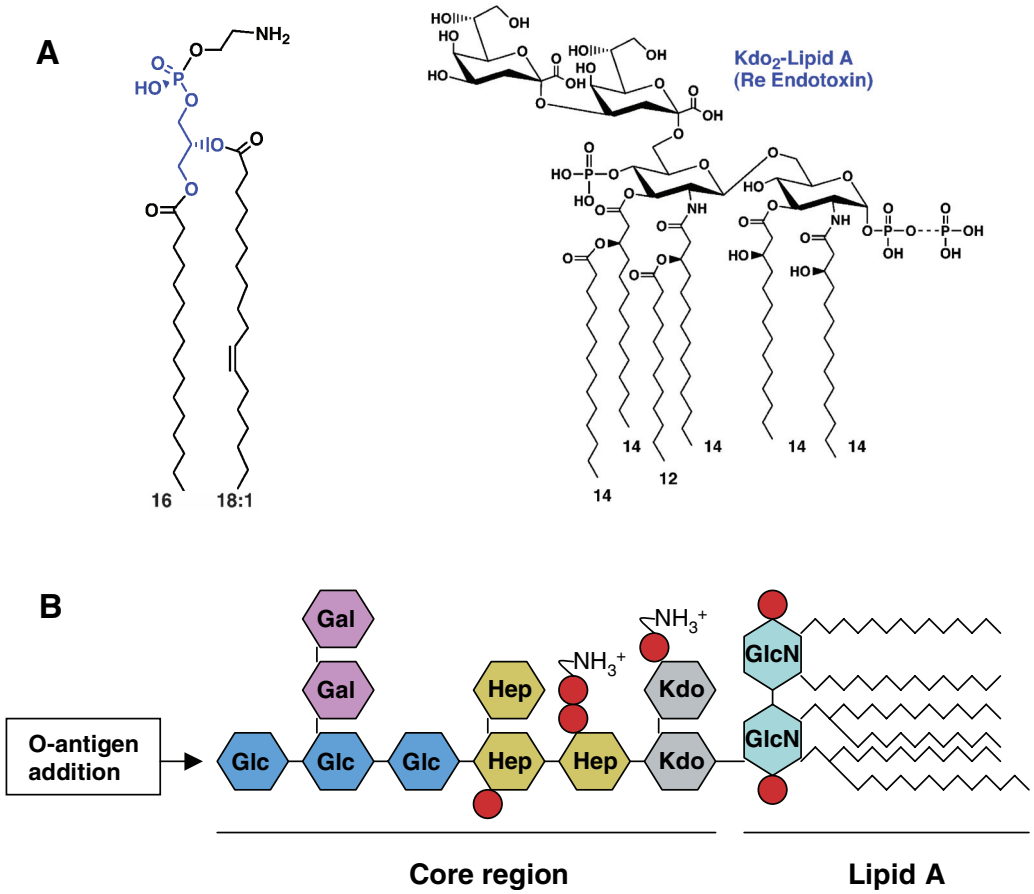

Fig. 2. A. Structures of the major *E. coli* membrane lipids PE and Kdo_2-lipid A.
B. Representative structure of *E. coli* LPS with core sugars shown. Phosphates are shown as red circles, ethanolamine by the curved line with an amine group, and sugars by hexagons. For simplicity, detailed information on linkages between sugars is not shown.

detail and in at least two cases (the fatty acid synthesis pathway and the LpxC deacetylase of the lipid A pathway) are promising targets for the development of novel antibacterial compounds (Onishi *et al*., 1996; Heath and Rock, 2004; McClerren *et al*., 2005). Ra-lipid A is the LPS found in common laboratory *E. coli* K12 strains and contains core oligosaccharide sugars covalently attached to lipid A (Fig. 2). LPS from many natural isolates additionally contains an O-antigen polymer ligated to the core region of LPS. Ra-lipid A is synthesized at the inner leaflet of the IM with the sugars donated by nucleotide-sugar precursors, while the O-antigen pentasaccharide repeat is added in the periplasm from an undecaprenol-pyrophosphate precursor (Raetz and Whitfield, 2002).

First step: across the IM. Model systems

Once the cytoplasmic steps of biosynthesis are complete, newly synthesized phospholipids and Ra-lipid A are transported across the IM bilayer. The mechanisms used to shuttle lipids across biological membranes (flip-flop) remain poorly understood. Transport of amphipathic lipids across a hydrophobic lipid bilayer is predicted to be thermodynamically unfavourable, requiring input of energy (Singer and Nicolson, 1972). Transport of lipids across bilayers has been studied in many excellent *in vitro* models. In this discussion, I will use the general term *flip-flop* to describe the outward movement of lipids across a bilayer. While this term does not discern outward from inward movement of lipids, only the former will be considered. Lipid flip-flop in model lipid bilayers is extremely slow, with half-lives on the order of hours to days, but is very fast in biological membranes, occurring in seconds to tens of seconds (Rothman and Kennedy, 1977). This has led to the conclusion that lipid flip-flop in biological membranes is protein catalysed. Several candidate proteins have been identified in eukaryotes that catalyse transbilayer movement of different classes of lipids (Daleke, 2003; Holthuis and Levine, 2005; Pohl *et al*., 2005). However, in prokaryotes, the only protein thus far identified with a proven role in lipid transport is MsbA (see below).

Differing conclusions have been reached with regard to energy requirements for IM lipid transport. In some sys-

tems, lipid flip-flop is ATP dependent but not in others. Work by deKruijff and colleagues suggests that lipid flip-flop in reconstituted vesicles is energy independent and requires only hydrophobic membrane-spanning α-helical peptides (Kol *et al*., 2004). In their studies, the authors tested the ability of membrane-spanning peptides to induce flip-flop of short chained, fluorescently labelled phospholipids (C6-NBD-PE and C6-NBD-PG) in artificial bilayers (Kol *et al*., 2001). They found that vesicles containing the model transmembrane α-helical peptide $GKKL(AL)_{12}KKA$ supported flip-flop of the short-chain lipids, whereas flip-flop was negligible in lipid vesicles alone. This work represents an interesting alternative to the concept that a dedicated protein machinery catalyses transbilayer lipid movement and that suggests this activity may be a general property of membrane proteins containing α-helical membrane-spanning domains.

Menon and colleagues have presented evidence for an ATP-independent flippase activity in membranes of Gram-positive bacteria (Hrafnsdottir and Menon, 2000) and this activity has also been detected in membranes from Gram-negative bacteria (Kubelt *et al*., 2002). Using di-C4-phosphatidylcholine as a substrate for the flippase assay, it was possible to solubilize, reconstitute and partially purify the activity. However, to date, a protein(s) has not been identified nor has a mechanism been established.

On the other hand, work with reconstituted ATP binding cassette (ABC) transporters from bacteria (Margolles *et al*., 1999) or humans (Romsicki and Sharom, 2001) has shown that they possess ATP-dependent phospholipid flippase activity *in vitro*. ABC transporter proteins use the energy of ATP hydrolysis to transport a variety of lipid and non-lipid substrates across membranes, and are present in all three domains of living organisms. Konings and colleagues have demonstrated (again using short-chain fluorescent phospholipid analogues) that purified, reconstituted LmrA, an ABC transporter from *Lactococcus lactis*, possesses phospholipid flippase activity *in vitro* that is dependent on exogenous ATP (Margolles *et al*., 1999). In a similar manner, reconstituted human p-glycoprotein/MDR1 (ABCB1) has been shown to possess ATP-dependent flippase activity with a wide variety of long- and short-chain phospholipid analogues (Romsicki and Sharom, 2001). While *in vitro* studies of phospholipid flip-flop activity have proven informative, caution should be used when extrapolating from these results to the *in vivo* situation, as they typically employ short-chain, water-soluble lipid analogues or contain other modifications that create structurally unique molecules that may differ in important ways from their naturally occurring counterparts.

In vivo studies and MsbA, a putative flippase

A large amount of genetic, structural and biochemical evidence implicates MsbA in IM transport of newly synthesized Ra-lipid A and probably phospholipids (Zhou *et al*., 1998; Doerrler *et al*., 2001; 2004; Reyes and Chang, 2005). MsbA was first identified by its ability to complement, when present in multiple copies, the growth defect of an *htrB* deletion mutant at elevated temperatures (Karow and Georgopoulos, 1993). HtrB is an acyltransferase involved in a late step of lipid A biosynthesis (Clementz *et al*., 1996) and mutants, though viable up to 33°C (Karow *et al*., 1991), produce underacylated LPS that is not transported efficiently to the OM at high growth rates (Zhou *et al*., 1998). MsbA is an essential IM protein, with mutations being lethal to most Gram-negative, and a member of the ABC transporter superfamily of proteins (Karow and Georgopoulos, 1993). MsbA homologues are found in nearly all species and include p-glycoprotein/MDR1 (ABCB1), a protein overexpressed in human carcinoma cells, and a contributor to the multidrug-resistance phenotype observed in many cancer cell lines. A role for MDR proteins, and other ABC transporters, in lipid trafficking in eukaryotes is well established (Pohl *et al*., 2005). Most notably, an MDR2 knockout mouse has a defect in phospholipid secretion into bile (Smit *et al*., 1993). Several MsbA crystal structures have been published and all suggest that the protein forms a homodimer in the IM with a chamber oriented towards the cytoplasm capable of accommodating lipid molecules (see below) (Chang and Roth, 2001; Chang, 2003; Reyes and Chang, 2005). Indeed, MsbA has served as a useful model providing structural insights into the mechanisms of action of other ABC transporters (Conseil *et al*., 2006).

A temperature sensitive *E. coli* mutant strain (WD2) containing a single amino acid substitution (A270T) in one of the membrane-spanning helices of MsbA was isolated and characterized (Doerrler *et al*., 2001). Upon inactivation of MsbA by a shift to the non-permissive growth temperature of 42–44°C, the mutant rapidly (within 30 min) accumulates newly synthesized LPS and phospholipids in the IM. This is accompanied by the formation of IM folds and invaginations visible by electron microscopy. Subsequent studies localized the accumulating lipids to the cytoplasmic leaflet of the IM (Doerrler *et al*., 2004). Topological analysis of lipids in a living cell is technically difficult and was done using different approaches for phospholipids and LPS. Membrane impermeable reagents that covalently modify the amine moiety of PE were less reactive with newly synthesized PE following inactivation of MsbA, indicating inaccessibility to these reagents and a cytoplasmic orientation. For analysis of the topology of newly synthesized lipid A, a strain was constructed that expresses the temperature-sensitive *msbA* allele in a polymyxin-resistant background (WD102). Polymyxin-resistant strains of *E. coli* and *Salmonella* covalently modify lipid A with cationic substituents

4-amino-4-deoxy-L-arabinose and phosphoethanolamine (Zhou *et al*., 1999; Trent *et al*., 2001). Both of these modifications are required for resistance to the antibiotic polymyxin (Lee *et al*., 2004; Breazeale *et al*., 2005). Importantly, the nature of the enzymes and substrates involved in these modifications strongly suggests that lipid A modification occurs in the *periplasm*, following IM flip-flop. Following inactivation of MsbA in WD102, newly synthesized lipid A was located in the IM and entirely unmodified with the cationic substituents, indicating that the lipid had not accessed the periplasmic compartment. This result strongly suggests that the newly synthesized LPS in this strain is oriented towards the cytoplasm. In these studies, the combined use of genetics and biochemistry has provided strong evidence that MsbA is required for transport of both LPS and phospholipids across the IM of *E. coli*.

To date, no *in vitro* system has been described to study the transbilayer movement of lipid A or any lipid A-like analogue. Purified hexaacylated lipid A, Kdo_2-lipid A and LPS have a stimulatory effect on the ATPase activity of the purified and reconstituted MsbA protein (Doerrler and Raetz, 2002), perhaps indicative of recognition and transport by the protein. Stimulation of this ATPase activity was only observed with hexaacylated lipid A species, and not with other LPS precursors such as tetraacylated lipid IV_A or diacylated lipid X.

It is important to stress at this point that, in spite of the above *in vivo* evidence, and *in vitro* evidence that purified and reconstituted MsbA exhibits a lipid-stimulated ATPase activity (Doerrler and Raetz, 2002), MsbA has never been shown to possess phospholipid (or lipid A) flip-flop activity *in vitro*. In fact, one study has reported that purified and reconstituted MsbA is not capable of promoting flip-flop of short-chain phospholipids in an ATP-dependent or -independent manner under conditions where membrane-spanning peptides were active (Kol *et al*., 2003). In addition, integral IM proteins leader peptidase and the potassium channel KcsA were able to induce lipid flip-flop, presumably due to the presence of membrane-spanning domains. Ironically, MsbA, a membrane protein containing six membrane-spanning helices in each monomer, was inactive in the same assay.

Furthermore, the *msbA* gene has been deleted from a capsule-deficient strain of *Neisseria meningitidis* (Tefsen *et al*., 2005a), an organism that does not require LPS for viability (Steeghs *et al*., 1998). The *msbA* deletion mutant is viable with only a slight growth defect and produces only small amounts of LPS but greatly increases amounts of phospholipids. Importantly, the mutant appears to produce an OM, as demonstrated by electron microscopy, which presumably consists mainly of phospholipids (though not directly shown by the authors due to technical problems separating IM and OM from the organism). The evidence from these two studies seems to suggest that MsbA is involved in LPS transport but not required for phospholipid transport across the IM.

How do we resolve this paradox? On the one hand, *msbA* conditional mutants convincingly accumulate phospholipids and LPS on the inner surface of the IM upon shift to non-permissive conditions (Doerrler *et al*., 2001; 2004). On the other hand, *in vitro* studies thus far are not consistent with a phospholipid flippase activity by the protein (Kol *et al*., 2003) and in *N. meningitidis* loss of MsbA does not appear to affect phospholipid transport to the OM (Tefsen *et al*., 2005a). One interpretation of the *in vitro* study failing to demonstrate an MsbA flippase activity is that additional undiscovered proteins in addition to MsbA may be required for efficient phospholipid transport across the IM *in vivo* and these are not present in the reconstituted systems. In bacteria, ABC transporter systems involved in import and export of substrates often utilize a soluble periplasmic binding protein component. In other words, MsbA may be necessary but not sufficient for flip-flop of phospholipids. To date, a protein that physically interacts with MsbA has not been identified. MsbA produced in the Gram-positive organism *L. lactis* is capable of conferring drug resistance to the organism (Woebking *et al*., 2005), suggesting that this property of the protein does not require additional host-specific factors. MsbA overexpression conferred an 86-fold resistance to erythromycin in *L. lactis* and transport of ethidium bromide and Hoechst 33342 was observed. Hoechst 33342 is an fluorescent, amphipathic drug transported by several MDR proteins (Margolles *et al*., 1999). Importantly, transport of Hoechst 33342 was inhibited by free lipid A with a K_i of 57 μM, indicating a direct interaction of lipid A with the protein and underscoring the relevance of the Gram-positive model for the study of drug transport properties of MsbA.

The results from the *N. meningitidis msbA* deletion strain are more difficult to reconcile with the *E. coli* mutants. In this organism, perhaps another transport protein or an entirely different mechanism is used to transport phospholipids across the IM. Details of the molecular mechanism of MsbA-dependent and -independent lipid transport in *E. coli* and other Gram-negative species await further biochemical and genetic studies.

Another possibility that cannot be discounted is that MsbA may only be required for the IM flip-flop of Ra-lipid A but not phospholipids. Phospholipids do accumulate in the IM of the MsbA conditional mutant WD2 at the non-permissive temperature (Doerrler *et al*., 2001; 2004). However, it is a formal possibility that this accumulation may be a secondary effect of a toxic accumulation of Ra-lipid A in the cytoplasm and phospholipids are not normally transported directly by the protein. Bioinformatic studies support this hypothesis. MsbA is only found in

Gram-negative bacteria that synthesize lipid A. The best MsbA homologues from Gram-positive bacteria, such as LmrA, have no greater identity to MsbA than the MDR proteins of mammalian species (roughly 30–35%), some of which may be involved in phospholipid transport. This is true even of *Deinococcus radiodurans* and the spirochaetes *Borrelia burgdorferi* and *Treponema pallidum*, organisms that possess OM but do not make lipid A.

MsbA: structural studies

Recent structural studies have shed light upon possible mechanisms of MsbA function. I will briefly describe some recent findings in this exciting field and direct readers to more detailed reviews for further information (Davidson and Chen, 2005). The recently reported structure of *Salmonella typhimurium* MsbA by Chang's group (Reyes and Chang, 2005) is regarded as a great improvement over the first two reported structures by the group (*E. coli and Vibrio cholera* respectively) (Chang and Roth, 2001; Chang, 2003) that were criticized as non-physiologic due to missing or misfolded nucleotide binding domains. All structures represent MsbA as a homodimer with six transmembrane helices in each monomer, a linker region and a cytoplasmic nucleotide-binding domain containing the highly conserved Walker motifs. The newest structure shows the NBD correctly folded in the post-hydrolysis state with bound ADP-vanadate (Fig. 3). In addition, Ra-LPS was included during the formation of crystals and is found bound to the protein on the periplasmic side of the membrane domain in a, presumably, 'post-flipped' conformation. These structures support the concept that MsbA is an ATP-powered 'pump' and sheds light upon the mechanism of action of other members of the MDR family of proteins.

The MsbA temperature-sensitive mutant WD2 contains the single amino acid substitution A270T (Doerrler *et al.*, 2001). Analysis of the location of the A270 residue in the newly published crystal structure of *S. typhimurium* MsbA (91% sequence identity to *E. coli* MsbA) shows that it lies at the dimer interface near the adjacent A270 residue (Fig. 3) at the periplasmic surface of the protein. The side-chain carbons of the two alanine residues are effectively in contact, only 3.28 Å apart. Threonines at this site, with its bulkier side-chain, may effectively destabilize the dimer at elevated temperatures and explain the temperature-sensitive nature of this mutation.

Electron paramagnetic resonance (EPR) was utilized to illustrate the dynamic nature of the MsbA power stroke (Dong *et al.*, 2005). In a heroic effort, the authors began

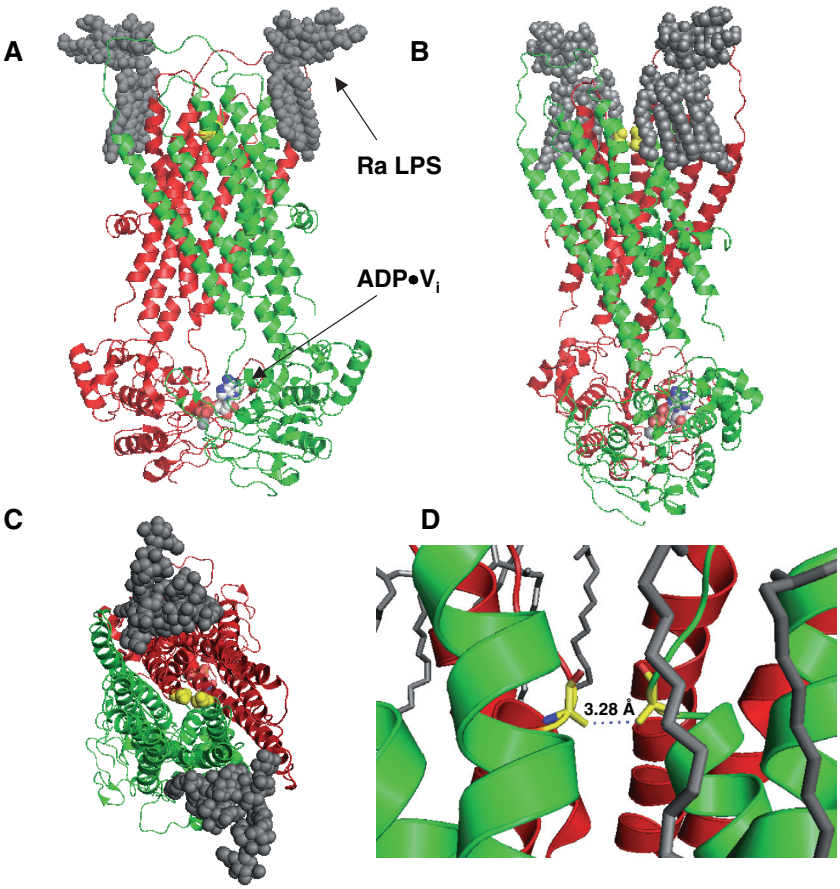

Fig. 3. Crystal structure of *S. typhimurium* MsbA highlighting the location of alanine 270, which is mutated to threonine in the MsbA temperature-sensitive mutant WD2 (Doerrler *et al.*, 2001). Analysis of the location of the A270 residue in the newly published crystal structure of *S. typhimurium* MsbA (91% identity to *E. coli* MsbA) (Reyes and Chang, 2005) shows that it lies at the dimer interface near the adjacent A270 residue at the periplasmic surface of the protein (yellow amino acid). The side-chain carbons of the alanine residues are effectively in contact, only 3.28 Å apart. A threonine at this site with its bulkier side-chain may destabilize the dimer at elevated temperatures and explain the temperature sensitive nature of this mutation.
A and B. View of dimer in the plane of the membrane.
C. View of MsbA dimer perpendicular to membrane from periplasmic surface.
D. Close-up of A270 residues showing distance between side-chain carbons. Co-ordinates were obtained from the RCSB Protein Data Bank (Berman *et al.*, 2000).

with a cysteine-less MsbA mutant and individually introduced engineered cysteine residues. More than 100 different mutants were produced containing single cysteines at different locations. The individual proteins were then expressed and purified, reconstituted into lipid bilayers, and labelled with spin label probes. Using site-directed spin labelling and EPR, it was possible to determine if an individual amino acid is buried within the protein, exposed to an aqueous environment, or exposed to the lipid bilayer. The authors demonstrated marked changes in conformation upon addition of ATP. Additionally, they confirmed many aspects of the crystal structures but, interestingly, their work shows that the periplasmic loops of MsbA are much more open to the aqueous environment than the crystal structures suggest. The approach has the advantage over crystallography that it is possible to study the protein reconstituted in its natural bilayer environment. The only criticism of this work is that while the mutant proteins were tested for ATPase activity (and some only had only 10% of wild type activity), none were tested for *in vivo* function by their ability to complement an *msbA* chromosomal deletion.

Across the great divide: the periplasm

Following transport across the IM, proteins and lipids must move to the OM and, in the case of LPS, to the outer leaflet of the OM (Fig. 1). The mechanisms by which nascent proteins, phospholipids and LPS are moved across the periplasm and are assembled into the OM remain poorly characterized. However, there do appear to be different requirements for movement of LPS and phospholipids. In the 1970s, Osborn and colleagues demonstrated that phospholipids were capable of moving back and forth between IM and OM of Salmonella (double arrow in Fig. 1) (Jones and Osborn, 1977). LPS did not show such behaviour in these experiments.

More recently, de Cock and colleagues reported an *in vitro* system for studying transport of newly synthesized phospholipids and LPS from spheroplasts to the OM (Tefsen *et al*., 2005b). They reported that transport of newly synthesized LPS to the OM was dependent only upon a functional copy of MsbA, and no soluble periplasmic components were required. In contrast, newly synthesized phospholipids were not transported to the OM of spheroplasts, indicating that a soluble periplasmic intermediate may be required for this step. It was speculated that LPS might be transported to the OM via points of contact between the IM and OM. The molecular nature of these points of contact is currently unknown but this observation is reminiscent of the Bayer's bridges first observed by electron microscopy in the 1960s (Bayer, 1968). These EM data suggest that points of physical contact may exist between the IM and OM in the periplasmic space that could be used for transport of molecules between the membranes. Amazingly, newly synthesized LPS was reported to appear in patches in the OM that were adjacent to zones of adhesion between the two membranes (Muhlradt *et al*., 1973). The existence of these membrane contacts was called into question by Kellenberger using improved cryofixation techniques and it is not clear if they represent actual physical structures or are artifacts of the cell fixation process used in electron microscopy (Kellenberger, 1990; Bayer, 1991). At any rate, the *in vitro* system and fascinating results reported by de Cock and colleagues (Tefsen *et al*., 2005b) allow for the first time a biochemical approach to study the movement of phospholipids across the periplasm.

Delivery of LPS to the outer surface of the OM

Incorporation into the inner leaflet of the OM presumably completes the journey for OM phospholipids. Once these molecules are assembled into the inner leaflet of the OM, they can only be transported back and forth between the IM and OM, as reported by Osborn and colleagues (Jones and Osborn, 1977), or diffuse laterally in the inner leaflet of the OM (Fig. 1). Agents that disrupt the OM, such as the calcium chelator EDTA, drive phospholipids to the outer leaflet of the OM where they can become substrates for the OM lipid A acyltransferase PagP (Jia *et al*., 2004), which plays a role in pathogenesis and resistance to antimicrobial peptides (Bishop, 2005).

In contrast to phospholipids, LPS needs to be flipped across the OM in a process analogous to IM LPS flip-flop carried out by MsbA. However, no obvious energy source is available to drive this process in the periplasm. Recent publications have identified two OM proteins potentially involved in lipid transport to the OM, YaeT (or Omp85) and Imp (OstA). *E. coli* YaeT is encoded by an essential gene located near minute 4 of the chromosome just upstream of genes involved in LPS and phospholipid biosynthesis. Depletion of Omp85 from *N. meningitidis* has been reported to result in loss of lipid targeting to the OM (Genevrois *et al*., 2003). However, this result has been disputed by other groups that have shown that depletion of Omp85/YaeT results in a loss of OM protein targeting and oligomerization (Voulhoux *et al*., 2003; Doerrler and Raetz, 2005; Werner and Misra, 2005) but has minimal effects on lipid transport (Doerrler and Raetz, 2005). *YaeT* is present in essentially all Gram-negative genomes sequenced to date including those organisms that do not synthesize LPS such as *D. radiodurans* and the spirochaetes *B. burgdorferi* and *T. pallidum*, consistent with a role in protein targeting to the OM. In addition, YaeT has sequence similarity to Toc75 and Sam50 involved in protein import into the chloroplast and mitochondria respectively, suggesting that aspects of OM assembly are

conserved across evolution (Gentle *et al*., 2005; Schleiff and Soll, 2005).

The only other protein currently suspected to play a role in LPS targeting to the OM is Imp/OstA (increased membrane permeability/organic solvent tolerance) or simply Imp. However, the evidence for a role of Imp in this process is indirect. Imp was first identified as a protein required for organic solvent tolerance (Sampson *et al*., 1989; Aono *et al*., 1994). Silhavy and colleagues demonstrated that *imp* encodes an essential OM protein in *E. coli* and that depletion of the protein results in abnormalities in OM assembly (Braun and Silhavy, 2002). Interestingly, depletion of Imp results in increased density of OM during sucrose gradient centrifugation, consistent with loss of lipid incorporation. More recently, Tommassen and colleagues have presented striking evidence that Imp is required for proper transport of LPS to the cell surface of *N. meningitidis* (Bos *et al*., 2004). This conclusion was based upon loss of surface accessibility of LPS to neuraminidase and loss of lipid A modification by the OM deacylase PagL. However, it was not possible to separate the IM from OM in this organism using sucrose gradients. Therefore, it is not possible to determine exactly where the LPS accumulates, information needed to assign a role to the protein in lipid trafficking. Interestingly, *imp* and *msbA* (see above), as well as the genes involved in LPS biosynthesis, are all *nonessential* genes in *N. meningitidis*. This is in contrast to *E. coli* and all other Gram-negative strains tested to date. The first gene of lipid A biosynthesis, *lpxA*, can be deleted from *N. meningitidis*, thereby blocking LPS synthesis but not affecting viability (Steeghs *et al*., 2001). The OM protein profile was unaltered from wild type in the *lpxA* mutant strain. It is thought that phospholipids can functionally replace LPS in these strains. These results suggest marked differences in envelope biology exist between species of Gram-negative bacteria.

More recently, the laboratories of Silhavy and Kahne found, applying chemistry and genetics in a technique termed 'chemical conditionality', that strains containing loss of function alleles of the gene *yfgL* in addition to an *imp* mutation (containing a 23-codon deletion) were resistant to certain antibiotic compounds to which the *imp* mutant itself was hypersensitive (Ruiz *et al*., 2005). YfgL was in turn found to be an OM lipoprotein associated with a heterooligomeric OM complex containing YaeT and two other lipoproteins (but not Imp) (Wu *et al*., 2005; Silhavy *et al*., 2006). While these studies shed light on OM biogenesis with regard to protein trafficking, the role of these proteins in lipid trafficking was not addressed. The exact mechanism of transport of LPS across the OM remains to be established but the aforementioned studies clearly suggest an important role for Imp in this process.

Outlook

Much remains to be learned about the process of lipid movement through the Gram-negative cell envelope and several controversies remain to be settled. First, what is the true role of MsbA? While it is implicated in transmembrane movement of both LPS and phospholipids, *in vitro* systems and the *N. meningitidis msbA* knockout seem to suggest differing mechanisms for the transport of these two classes of molecules across the IM. Second, what are the energy requirements for transport across the IM? The involvement of the ABC transporter MsbA suggests that ATP is required. However, past studies have reported that disruption of the proton motive force under conditions that do not deplete ATP synthesis inhibits lipid A-core transport to the OM (McGrath and Osborn, 1991). Other studies have concluded that energy is not required for phospholipid transport across the IM, which needs only membrane-spanning helices of integral membrane proteins (Kol *et al*., 2004). Finally, the molecular requirements and mechanisms for lipid translocation across the periplasm and across the OM are all but unknown. What proteins are required for these processes and what is the role of Imp in this process?

While no specific inhibitors of the process of lipid export are known, this pathway represents a potential target for the development of novel antibiotics. Recall that MsbA has extensive sequence identity to mammalian p-glycoprotein/MDR1. P-glycoprotein was first identified by virtue of its overexpression in multidrug-resistant cancer cell lines (Chen *et al*., 1986; Gros *et al*., 1986). There is currently great interest by the pharmaceutical industry in identifying *in vivo* inhibitors of p-glycoprotein in order to reduce drug excretion from multidrug-resistant cancer cells and to improve treatment options of cancer patients (Ambudkar *et al*., 1999). In fact, clinical trials of candidate inhibitors have been conducted. Lead compounds identified in these types of screens could represent a starting point in the search for *in vivo* modulators of the activity of MsbA and, indeed, novel classes of antibiotics.

In conclusion, the biogenesis of the Gram-negative envelope is an intriguing system for the study of lipid trafficking. These studies have brought into play the tools of genetics, biochemistry and structural biology. While we have learned much about this system in the past few years, many questions remain stubbornly unanswered.

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